Megakaryocyte-matrix interaction within bone marrow: new roles for fibronectin and factor XIII-A

Alessandro Malara¹, Cristian Gruppi¹, Paola Rebuzzini², Livia Visai³, Cesare Perotti⁴, Remigio Moratti¹, Cesare Balduini¹, Maria Enrica Tira¹, Alessandra Balduini¹-⁵*

¹Department of Biochemistry; ²Department of Animal Biology; ³Center for Tissue Engineering (C.I.T.); ⁴Immunohaematology and Transfusion Service, Apheresis and Cell Therapy Unit; IRCCS San Matteo Foundation, University of Pavia, Pavia, Italy; ⁵Department of Biomedical Engineering, Tufts University, Medford, MA, USA

Running Title: Megakaryocyte-matrix interaction.

*Corresponding author: Alessandra Balduini (alessandra.balduini@unipv.it), Biotechnology Research Laboratories, Department of Biochemistry, University of Pavia, IRCCS San Matteo Foundation, Pavia, Italy. (alessandra.balduini@tufts.edu), Department of Biomedical Engineering, Tufts University, Medford, MA, USA.
Abstract

The mechanisms by which megakaryocytes (Mks) differentiate and release platelets into circulation are not well understood. Growing evidence indicates that a complex regulatory mechanism, involving Mk-matrix interactions, may contribute to the quiescent or permissive microenvironment related to platelet release within bone marrow. To address this hypothesis, in this work we first demonstrated that human Mks express and synthesize cellular fibronectin (cFN) and transglutaminase FXIII-A. Thereafter, we proposed that these two molecules are involved in a new regulatory mechanism of Mk-type I collagen interaction in the osteoblastic niche. In particular, we demonstrated that Mk adhesion to type I collagen promoted Mk spreading and inhibited proplatelet formation through the release and relocation to the plasma membrane of cFN. This regulatory mechanism was dependent on the engagement of fibronectin receptors at the Mk plasma membrane and on FXIII-A transglutaminase activity. Consistently, the same mechanism regulated the assembly of plasma FN (pFN) by adherent Mks to type I collagen. In conclusion, our data extend the knowledge of the mechanisms that regulate Mk-matrix interactions within bone marrow environment and could serve as an important step for inquiring into the origins of diseases, such as myelofibrosis and congenital thrombocytopenias that are still poorly understood.
Introduction

Hemopoietic stem cells reside in bone marrow (BM) specialized niches that dictate how they differentiate, proliferate, mature and enter the peripheral circulation. Megakaryocyte (Mk) maturation and platelet generation is consequent to Mk migration from the osteoblastic to the vascular niche, where Mks extend proplatelets (PPF) and newly generated platelets are released into the bloodstream.

The characteristics of the microenvironment surrounding Mks play an important role in the regulation of platelet production within BM. In particular, the interaction of Mk with different extra-cellular matrices (ECMs), that fill the BM spaces, seems to orchestrate their maturation in specific sites. It has been demonstrated that interactions of primary human Mks with matrices supposed to fill the vascular niche, such as fibrinogen (FBG) or von Willebrand Factor (vWF), is able to sustain Mk maturation and PPF, while type I collagen, in the osteoblastic niche, totally suppresses these events and prevents premature platelet release. The negative regulation of PPF by type I collagen is mediated by the interaction with integrin alpha2beta1, and involves the Rho/ROCK pathway. However the exact subsequence of events that determine Mk interaction with ECM and therefore their regulation, is not completely understood. Recent works have demonstrated that the encounter between a cell and an adhesive substrate determines an initial passive interaction characterized by cell adhesion and spreading, followed by an active stage that involves actin polymerization and myosin contraction triggered by the activation of a complex cascade of signaling events. Interestingly, nothing is known about the mechanisms that regulate the dynamic of Mk-ECM interaction within the BM environment.

Recent works have demonstrated that fibronectin (FN) may represent a new regulator of Mk maturation and platelet release. FN are produced from a single gene, but alternative splicing of their pre-mRNA leads to the creation of several isoforms. The amino acid sequence variations are the consequence of the alternative processing of a single primary transcript at three conserved sites: extra domain B (EDB) or extra type III homology B (also called EIII-B), extra domain A
(EDA or EIII-A) and type III homologies connecting segment (IIICS; V region in rat). The EDA and EDB are single exons coding for single type III repeats that are included or excluded from the FN mRNA by exon skipping. EDB FN isoform protein was detected in guinea pig and human Mks, but its function remains poorly understood.

FN and type I collagen are cross-linked, at cellular site, by activated transglutaminases (TGs). The TG family consists of nine known members of whom at least three are expressed in the vascular system: type 1 TG, type 2 TG and Factor XIII. Recent data suggest that TGs play a role in several other processes such as cell differentiation, adhesion and migration. In platelets TG activity depends on the presence of FXIII-A and a significant reduction of soluble fibrinogen binding in response to thrombin receptor agonist peptide (TRAP) was observed in FXIII-deficient platelets. In contrast, platelet FXIII-A has been proposed to negatively regulate alphaIbbeta3 integrin function in collagen-adherent platelets.

In this study, we showed, for the first time, that human Mks express and synthesize cellular fibronectin (cFN). Therefore, we propose that Mk adhesion to type I collagen prevents PPF and sustains Mk spreading through a mechanism that involves FN, membrane receptors and FXIII-A TG activity. This mechanism seemed to be mediated by the exposure of cFN to the cell membrane and maintained by FN polymerization catalyzed by FXIII-A. These data address a new role to FN that, upon specific activation, could be released and thereby modulate Mk interaction with ECMs. In this context FXIII-A catalyzed FN cross-linking at cellular sites, stabilized FN assembly and promoted the organization of ECMs. These experiments open new insight into the understanding of the role of force development and cell spreading on Mk-ECM interaction that are still unknown.

Materials And Methods

Reagents and antibodies.

Type I collagen and human plasma fibronectin were purified as previously described. Human plasma fibrinogen was from Calbiochem (La Jolla, CA, USA). Human factor XIII was from...
Haematologic Technologies Inc. (Vermont, USA), Biotin-cadaverine was from Anaspec (Fremont, CA, USA). Iodoacetamide, cystamine, monodansylcadaverine, thrombin from human plasma, blebbistatin and fluorescein isothiocyanate were from Sigma-Aldrich (Milan, Italy). The following antibodies were used: rabbit polyclonal anti-plasmatic and cellular fibronectin was a kind gift of Dr. Visai 28, mouse monoclonal anti-fibronectin (clone IST-4), mouse anti-EDA+ fibronectin (clone FN-3E2), phalloidin-TRITC, mouse anti-beta actin (clone AC-15), mouse anti α-tubulin (clone DM1A) and FITC-anti mouse IgM were from Sigma-Aldrich, mouse monoclonal anti-Factor XIIIa Ab-1 (clone AC-1A1) was from LabVision, mouse monoclonal anti CD49b (clone HAS-4) was from Abcam (Cambridge, UK), mouse monoclonal anti CD49e (clone SAM-1), anti-CD49d (clone P1H4) were from Chemicon (Millipore, Billerica, MA, USA), anti-CD49e (clone IIAn1) was from BD Bioscience (Milan, Italy). Mouse monoclonal anti-CD61 (clone SZ21) was from Immunotech (Marseille, France). Alexa conjugated antibodies were purchased from Invitrogen (Milan, Italy) and colloidal gold conjugated goat antibody to rabbit IgG with 40nm particle size was purchased from EY Laboratories Inc (San Mateo, CA, USA).

**Human cord blood derived Mks.**

Human cord blood was collected following normal pregnancies and deliveries upon informed consent of the parents, according with the ethical committee of the IRCCS Policlinico San Matteo Foundation and in accordance with the Principles of the Declaration of Helsinki. CD34+ cells were separated and cultured as previously described. 7,29

**Evaluation of PPF and cell spreading in adhesion to different matrices.**

To analyze PPF onto different adhesive substrates, 12 mm glass coverslips were coated with fibrinogen or type I collagen as described. 7 Cells at day 13 of culture were harvested and allowed to adhere for 3 or 16 hours (1 x 10^5 cells/well) at 37°C and 5% CO₂. Cell spreading was determined as previously described. 7 In some experiments, Mks were harvested and pre-incubated with specific
inhibitors or antibodies for 30 minutes at 37°C used at the following final concentrations: 10 μM iodoacetamide, 10 μM cystamine, 0.1 mM monodansylcadaverine, 10 μg/ml anti CD49d, 10 μg/ml anti CD49e (clone IIA1), 1mM RGDS, 50 μM blebbistatin.

Immunofluorescence and confocal microscopy analysis.

Preparation of samples for analysis of subcellular localization of cellular fibronectin in activated Mks was performed as previously described and Mks were stained with rabbit polyclonal anti-fibronectin antibody 10μg/ml and goat anti-rabbit secondary antibody, while distribution of CD49b and CD49e were analyzed with an anti CD49b (clone HAS-4) and anti-CD49e (clone SAM-1) both diluted 1:100. FXIII-A was stained in non permeabilized cells using antibody clone AC-1A1 diluted 1:100. Mks extending proplatelets were stained with a mouse monoclonal anti α-tubulin diluted 1:700.

Analysis by conventional fluorescence and confocal microscopy were performed with an Olympus BX51 (Hamburg, Germany) microscope, using a 63x/1.25 or a 100x/1.30 UplanFl oil-immersion objective and a TCS SPII Confocal Laser Scanning Microscopy system (CLSM) (Leica, Heidelberg, Germany), equipped with a Leica DM IRBE inverted microscope as previously described.

RT-PCR and qRT-PCR.

CD61+ Mks at day 13 of culture were separated by immunomagnetic beads technique (Miltenyi-Biotec, Bergisch Gladbach, Germany) and total cellular RNA was extracted using Mammalian GeneElute Total RNA Kit (Sigma-Aldrich, Milan, Italy) and reverse transcribed to cDNA using MuLV reverse transcriptase (Applera, Monza, Italy) by following manufacturer’s instructions.
For RT-PCR detection of fibronectin and its splice variants EDA+ and EDB+ fibronectin, one third of the resulting cDNA was amplified using the following primers: 5’ GGAGAGAGTCAGCCTCTGGTTCAG 3’ and 5’ TGTCACCTGGGCGCTCAGGCTTGTG 3’, that are located in the exons III-11 and -12 flanking the EDA region and amplifies both EDA+ fibronectin and EDA-fibronectin mRNA; 5’ CGGCCTGGAGTACAATGTCAGTGT 3’ and 5’ CAGGTGACACGCATGGTGTCTGGA 3’, that are located in the exons III-7 and -8 flanking the EDB region and amplifies both EDB+ fibronectin and EDB-fibronectin mRNA; 5’ GCCTGGTACAGAATATGTAGTG 3’ and 5’ ATCCCAGCTGATCAGTAGGCTGTTG 3’, that amplifies a fibronectin region (III-9) which does not undergo alternative splicing, common to all fibronectin mRNA. The amplification reaction was performed in 25 µl using an Applied Biosystems GeneAmp 9700 thermocycler (Applied Biosystem) and twenty microliters of PCR products were electrophoresed in a 2% agarose gel stained with ethidium bromide.

For tranlglutaminases expression, one tenth of the resulting cDNA was amplified using the following set of primers: TGM1 (NM_000359) 5’ CCATGATTCTGTCTGGAACT 3’ and 5’ CACAGAGACCATCAGATCCT 3’, TGM2 (NM_004613.2) 5’ ATGAGAAATACCGTGACTGC 3’ and 5’ GGCATATTTTGCTCACTAGC 3’, TGM3 (NM_003245.3) 5’ GGATAGCGTCTTTATGGGTA 3’ and 5’ GAGATTTCCGCTGTGTCAT 3’, FXIIIA1 (NM_001994) 5’ AAGGTGTCATGAAATCAAGG 3’ and 5’ TGGTCTCTGTTACTCCAGGAC 3’, FN1 (NM_212482.1) 5’ GAACGTATGATGCCGACCAGAA 3’ and 5’ CTGATCTCAGAATTCGACGTATGATGCCGACCAGAA 3’. Twenty µl of the PCR products were loaded on ethidium bromide stained 1% agarose gel.

For quantitative expression analysis of TGs, a Real Time PCR was performed using the Rotorgene 6000 (Eurogentec, Milan, Italy). One twentieth of the resulting cDNA was amplified in triplicate utilizing the MESA GREEN qPCR MasterMix Plus for SYBR assay no ROX sample
(Eurogentec, Milan, Italy). The Rotorgene 6000 Series Software 1.7 was used for the comparative concentration analysis.

**Western Immunoblotting.**

Cell culture supernatants were collected and centrifuged for 10 minutes at 13,000 rpm to pellet floating cells. Adherent Mks were washed with PBS and lysed with RIPA buffer 1X (PBS with Nonidet P-40 2%, sodium deoxycholate 1%, SDS 0.2% and protease inhibitors). Protein concentrations of both the supernatant and cellular fractions were determined using the bicinchoninic acid (BCA) assay (Pierce, Milan, Italy). Equal amounts of proteins were then used for SDS-PAGE immunoelectrophoresis: secreted FN was assessed in the suspension medium by immunoprecipitation and visualized in a 7.5% reduced gel with antibody anti-fibronectin, clone IST-4, diluted 1:1000, and detected by a horseradish peroxidase (HRP)-labeled secondary antibody using enhanced chemiluminescence (ECL) (Pierce, Milan, Italy). Proteins form adherent Mks were separated by SDS-PAGE and immunostained with antibody anti-beta actin diluted 1:10000.

**Flow cytometry**

For DNA content analysis, low ploidy Mks at day 9 of culture were split and incubated with 25 μg/ml of type I collagen, FNG or PBS as control and reseeded for additionally 4 days in fresh medium. At the end of culture, cells were harvested and fixed with cold ethanol 70% and freeze over night at -20 C°. Subsequently, cells were stained with a FITC-conjugated antibody against human CD41 (clone HIP8, BioLegend, California, USA) for 30 minutes on ice at dark and with 50 μg/ml Propidium Iodide (PI) (Sigma, Milan, Italy) supplemented with 100 μg/ml RNase (Sigma, Milan, Italy) for 30 minutes at 37 C°. The ploidy distribution in the CD41+ population was determined by two-color flow cytometry (FACS calibur flow cytometer, BD Biosciences, San Jose’, CA, USA). A minimum of 20000 events were collected in the CD41+ gate. Off-line data analysis was performed using FCS Express 3.0 (DeNovo Software, Los Angeles, CA, USA).
Pull-down assay of GTP-loaded Rho.

RhoA activity assay was performed using a Rho activation assay kit (Upstate Biotechnology, Souffelweyersheim, France) following manufacturer instructions. In parallel, total amount of Rho was determined by immunoblotting.

Assembly of fibronectin by adherent Mks under static conditions.

Mks were incubated for 30 minutes at 37 °C with 25 μg/ml of FITC-labeled human plasma fibronectin and seeded for additional 16 hours on matrices-coated coverslips and immunofluorescence microscopy was performed as above described.

DOC extraction of fibrillar deposition of fibronectin.

Mks were plated on different matrices coated well plates for 16 hours in the presence of 25μg/ml human plasma fibronectin (pFN) at 37°C. Conditioned medium was then removed and matrix was solubilized using 200 μl DOC lysis buffer (PBS, deoxycholic acid 0.1%, EDTA 2mM). After centrifugation, the DOC-insoluble pellet was solubilized in 25 μl of 2% SDS, 20 mM Tris-HCl, pH 8.8, 2 mM PMSF, 2 mM iodoacetic acid, 2 mM N-ethylmaleimide and 2 mM EDTA. Equal volumes of DOC-insoluble samples were analyzed by SDS-PAGE using 5% polyacrylamide gels. Samples were immunoblotted and incubated with IST-4 anti-fibronectin monoclonal antibody. To ensure the same number of cells in the experiments, in a parallel sample, cells were scraped and proteins separated by SDS-PAGE and then incubated with anti-beta actin antibody. In some experiments, DOC insoluble matrix, was visualized by immunofluorescence using the rabbit polyclonal anti-fibronectin antibody, followed by an appropriate secondary antibody.

TG activity associated with Mk cell surface.
TG activity was measured by the incorporation of biotinylated cadaverine into fibronectin. For this assay, 96 well plate were pre-coated with human pFN (5 μg/well) and cells were then plated at a density of 2 x 10^5 cells/ml in the presence of 0.1 mM biotinylated cadaverine. Cells were allowed to adhere for 16 hours at 37°C. As a negative control, pFN coated 96-wells were incubated with medium containing 0.1 mM biotinylated cadaverine alone. In parallel samples, cells were pretreated with TGs activity inhibitors, such as iodoacetamide and cystamine 10μM for 15 minutes. The detergent solution consisting of 0.1% DOC in PBS was then added to each well and incubated for 20 minutes. The supernatant was discarded, and the remaining fibronectin layer washed three times with Tris-HCl. Reaction was blocked with 3% BSA in Tris-HCl and the incorporated biotinylated cadaverine was revealed with a 1:5000 dilution of peroxidase conjugate avidin after 1 hour incubation at 37°C. 200 μl of substrate solution (a mixture of H₂O₂ and tetramethylbenzidine) was then added for 20 minutes at RT. Color development was stopped by adding 50 μl stop solution and optical density measured with an ELISA plate reader (Biorad, Milan, Italy) set at 450nm.

**Intracellular TGs activity in human Mks.**

Transglutaminase activity was measured by the method of Jayo A. et al. Briefly, 1x10^6 Mks were lysed on ice for 20 minutes in 10 mM Tris-HCl pH 7.4, containing 0,1% Triton X-100, 140 mM NaCl, 0.4 mM PMSF and 1 mM DTT. After clearing by centrifugation, 10 μg of proteins were incubated with 400 μM biotinylated cadaverine with or without 5 mM EDTA and DMSO as negative control. After 1 hour at 37°C the reaction was stopped by adding loading buffer, then resolved in 10% SDS PAGE under reducing conditions and transferred to PVDF membranes. The membrane were incubated with peroxidase-conjugated avidin (1:5000 dilution) and visualized using the ECL chemiluminescent system. To analyze TPO effect on TG activity, cells were seeded in the presence of different concentrations of TPO (0, 50, 100 ng/ml) for 2 hours and then treated as above described.
Scanning Electron Microscopy (SEM) and immunogold.

Samples were fixed with 2.5% glutaraldehyde solution in 0.1 M Na-cacodylate buffer for 1 h at 4°C. Immunogold staining of fibronectin was performed incubating the cells for 2 hours with a polyclonal anti-fibronectin antibody followed by 1 hour incubation with a goat anti-rabbit secondary antibody conjugated with 40 nm gold beads and then dehydrated at room temperature in a gradient ethanol series up to 100%. Negative control were performed omitting primary antibody. The specimens were mounted on aluminum stubs, sputter coated with gold and then observed with a Leica Cambridge Stereoscan 440 microscope (Leica Microsystems, Bensheim, Germany) at 17.5 kV and a magnification of 5.00 K X.

Statistics.

T test was used to analyze data, with a significant difference set at p < 0.05. Data are presented as mean ± S.D of at least three different experiments.

Results

Human megakaryocytes express and synthesize cFN

Mks were derived from human umbilical cord blood progenitor cells and expression of cFN, along with the two isoforms EDA and EDB, were analyzed by RT-PCR. As shown in Figure 1A EDA fibronectin RT-PCR revealed a 374 and 104 bp amplification products corresponding respectively to EDA+ fibronectin and EDA spliced fibronectin mRNA sequence, while EDB RT-PCR revealed a 413 and 143 bp products corresponding to the EDB+ fibronectin and spliced EDB isoform, the RT-PCR for total fibronectin (FN1) revealed a 419 bp product. Further, cFN was also detected in Mk cytoplasm by a polyclonal antibody created in our laboratory (Figure 1B) and a specific antibody against the EDA isoform (clone 3E2) (Figure 1C). Finally, cFN relocated to Mk membrane upon
thrombin stimulation (Figure 1D). Overall, these results demonstrated that cFN was expressed by human Mks with a prevalence of the EDA+ isoform and was actively involved in Mk activation.

**cFN differently modulates Mk adhesion to bone marrow ECMs**

In order to analyze the role of cFN in regulating Mk-ECM interaction, mature Mks were plated on two different bone marrow ECMs: type I collagen, known to inhibit Mk maturation and PPF through the Rho-ROCK pathway, or FBG, known to support Mk maturation and PPF. After three hours incubation, about 25% of Mks were spread on both ECMs (Figure 2A), however, prolonging incubation at sixteen hours, Mks on FBG returned round and started to extend proplatelets (Figure 2B), while Mks on type I collagen remained spread and did not proceed on maturation (Figure 2A). To evaluate the effect of these ECMs on Mk differentiation, low ploidy Mks at day 9 of culture were also cultured with type I collagen or FBG for 4 days, but no significant variation in ploidy level was observed (Figure S1). In order to confirm the involvement of the alpha2beta1 in type I collagen dependent spreading, pull-down experiments were performed to evaluate the quantity of GTP-loaded Rho in Mks adherent to type I collagen using the GST-RBD (a fusion protein containing Rho-binding domain of Rhotekin), which selectively binds to the GTP-bound form of RhoA. Levels of GTP-bound RhoA increased upon adhesion of Mks to type I collagen with respect to Mks maintained in suspension (Figure S2).

Interestingly, upon adhesion to type I collagen, and not FBG, relocation of cFN to the plasma membrane was observed after three hours incubation and maintained thereafter (Figure 2C). Moreover, upon Mk adhesion to type I collagen, and not FBG, cFN was also released in the supernatant as revealed by western blotting performed upon immunoprecipitation (Figure 2D). Finally, Scanning Electron Microscopy (SEM) analysis confirmed that Mks on type I collagen, after three hours incubation, started to accumulate cFN on plasma membrane as revealed by immunogold staining (Figure 2E).
Further, co-distribution analysis of alpha 5 (CD49e) and alpha2 (CD49b) subunits with cFN demonstrated a strict association between alpha5 subunit with membraneous cFN and, to a lesser extent, with alpha2 subunit in Mks spread on type I collagen (Figure 3A). Further, Mk spreading was evaluated, on type I collagen, upon incubation with monoclonal antibodies anti-CD49e (clone SAM-1), anti-CD49d (alpha4 subunit) (clone P1H4) or their combination. Both antibodies determined a significant decrease in Mk spreading of about 50% with respect to control Mks incubated with a non related IgG antibody, and this effect was amplified by antibodies combination (Figure 3B). Consistently, Mk spreading was also decreased upon incubation with RGDS peptide (Figure 3B).

**Fibronectin assembly is dependent on Mk interaction with type I collagen**

Since relocation on the plasma membrane and secretion of cFN resulted to be dependent on the interaction of Mks with type I collagen, Mk ability of organizing pFN in an insoluble matrix was also explored. Mature Mks were plated either on type I collagen or FBG and let to adhere sixteen hours previous to incubation with fluorescein isothiocyanate (FITC)-labeled fibronectin. As observed for cFN, only Mks plated on type I collagen, and not on FBG, assembled FITC-FN as revealed by immunofluorescence analysis (Figure 4A). Moreover analysis of pFN assembly after deoxycholic acid (DOC)-treatment revealed the presence of fibrillar pFN only when Mks were plated on type I collagen (Figure 4A). Consistently immunoblot analysis revealed the presence of fibrillar pFN in the DOC-insoluble fraction in Mk-type I collagen, and not in Mk-FBG co-cultures, while pFN was present in the soluble fractions of both co-cultures (Figure 4B).

In order to demonstrate that FN assembly was strictly dependent on integrins binding and cell contractility, in some experiments, pFN was added to Mk cultures on type I collagen, as previously described, upon either incubation with RGDS, a synthetic peptide containing the RGD sequence that inhibits integrin-related functions, or treatment with 50 μM blebbistatin, a selective antagonist
of myosin IIA ATPase activity. Figure 4C shows an important decrease of matrix pFN assembly in the presence of both RGDS and blebbistatin with respect to control.

Finally, to verify the incorporation of endogenous EDA+ FN during pFN assembly, we performed experiments as above and stained FN assembly with both the antibody against the EDA isoform and the polyclonal antibody against FN. Figure 4D shows that pFN and cFN EDA+ isoform co-localized in the FN matrix assembly.

**Expression and activity of transglutaminases in human Mks**

In order to search possible mechanisms underlying matrix FN assembly by Mks, the transglutaminases (TGs) expression and localization were analyzed. Among the most well characterized members of the TGs family, only FXIII-A resulted highly expressed by Mks as verified by RT-PCR (Figure 5A) and qRT-PCR. Quantification of different TGs mRNA levels by qRT-PCR demonstrated that type 1 TG (TG1) and type 2 TG (TG2) expressions were barely detected, type 3 TG (TG3) was not expressed, while FXIII-A was easily amplified (data not show). Consistently, FXIII-A was also detected at protein level by western blot analysis, using FXIII-A from human plasma as control (Figure 5B). Intracellular TG activity of FXIII-A in Mks was demonstrated by incorporation of biotinylated cadaverine, a small molecule amine-donor TG substrate, into different proteins in Mk lysate with the method of Jayo et al.26, and this activity was completely reverted upon calcium sequestering with EDTA and completely absent in the control DMSO (Figure 5C). Interestingly, TPO concentration was inversely associated with FXIII-A activity, as a reduction of biotinylated proteins was observed when Mks were incubated for two hours with increased TPO concentrations (Figure S3).

Further, cell surface associated TG activity, presumably due to FXIII-A, was shown by the incorporation of biotinylated cadaverine into a layer of fibronectin and detected by ELISA assay upon adhesion of Mks for sixteen hours upon fibronectin (Figure 5D). Decreased FXIII-A activity
was shown upon incubation with TG inhibitors such as iodoacetamide (IAA) and cystamine (CYS) (Figure 5D).

**Function and localization of FXIII-A in human Mks**

In order to demonstrate FN-FXIII-A interaction, during the assembly of exogenous fibronectin, co-distribution of pFN and FXIII-A was analyzed. As shown in Figure 6A, pFN-FXIII-A co-localized on the plasma membrane of spread Mks on type I collagen after an incubation of sixteen hours (Figure 6A). To better understand the role of FXIII-A in regulating Mk function, Mk spreading on type I collagen was evaluated upon sixteen hours incubation with TGs inhibitors, such as IAA, monodansylcadaverine (MDC) and CYS. As shown in Figure 6B TG inhibitors determined a significant decrease in Mk spreading on type I collagen demonstrating the fundamental role of FXIII-A in stabilizing Mk cytoskeleton and in moderating Mk-type I collagen interaction. Consistently, in the presence of IAA Mks presented an evident reduction in actin stress fiber formation and lost their ability of assembling pFN with respect to control as revealed by immunofluorescence analysis (Figure 6C).

**Discussion**

The characteristics of the bone marrow environment play an important role in the regulation of Mk development and proplatelet formation. Type I collagen is known to inhibit proplatelet extension through the engagement of integrin alpha2beta19,12 and activation of the Rho/ROCK signaling cascade with the implication of myosin IIa.10,11 Despite the improvement in knowledge of the biochemical niche, little is known about the complex and dynamic regulation of Mk interaction with the BM-matrix environment.31 Demonstrating the expression of cFN and FXIII-A by human Mks, we propose a new, more complex, mechanism underlying Mk-type I collagen interaction in the osteoblastic niche (Figure S4).
In a previous paper, Schick et al.\textsuperscript{20} have shown that guinea pig Mk s synthesize the EDB FN isoform protein that is relocated to Mk membrane in response to thrombin. Therefore, the authors assumed that the redistribution of FN EDB on Mk surface may modulate Mk-matrix interaction. In this work we extend these observations by focusing on the expression and the function of the cellular FN isoforms in human Mks. We demonstrated, for the first time, that human Mks express cFN with a prevalence of the EDA isoform. Further, exposure on Mk plasma membrane of cFN was shown to occur only upon Mk adhesion on type I collagen and not on FBG. Moreover, relocation of cFN on Mk plasma membrane seemed to be related to a specific Mk function on type I collagen: in fact cFN was detected only on the plasma membrane of Mks spread on type I collagen. Interestingly, Mk spreading on FBG was not accompanied by cFN exposure on the plasma membrane and resulted in a shortened process over time that was replaced by PPF in 16 hours. Consistently, a recent work\textsuperscript{13} has described cell spreading as a dynamic process that lead to the generation of forces regulated by a complex cascade of signaling events. Therefore, these results may account for a mechanism in which cFN promotes the anchoring of Mks to type I collagen which results in activation of biochemical signalling and generation of contractile force that maintains Mk spreading over time. Further confirmation of these data was achieved by the evidence that only Mks on type I collagen were able to release cFN that bound to its integrin receptors on spread Mk plasma membrane. Finally, integrin alpha5beta1 and alpha4beta1 played a fundamental role as regulators of Mk function on type I collagen as their inhibition with specific antibodies, or incubation with the RGDS peptide, led to significant reduction of Mk spreading. All together these results pointed out a new role for cFN as modulator of biochemical and mechanical signalling underlying Mk-type I collagen interaction.

Beside the cellular form, FN exists in a soluble form in the blood plasma (pFN) that is known to enhance platelet thrombus formation on type I collagen.\textsuperscript{32} Further, several works have demonstrated that fibroblasts, endothelial cells and vascular smooth muscle cells secrete, bind, and assemble FN into fibrils in the ECM.\textsuperscript{33} Interestingly, FN fibrillogenesis is initiated by the binding
of the integrin alpha5beta1 to FN on cell plasma membrane,\textsuperscript{34,35} while FN fibril elongation is
dependent on interactions with type I collagen.\textsuperscript{36} Further, FN fibrillogenesis is dependent on cell
cytoskeleton reorganization triggered by activation of the Rho/ROCK signalling through myosin
IIA.\textsuperscript{37} In this context, the present work shows that also Mks can promote the assembly of
exogenous FN in a substrate dependent manner. FN-matrix assembly was detected only around
spread Mks on type I collagen as revealed after DOC-treatment by western blotting and
immunofluorescence techniques. Moreover, FN assembly was strictly dependent on forces
generated by contractility of Mk cytoskeleton as it was prevented by inhibition of actin-myosin
reorganization with the selective antagonist of myosin IIA ATPase activity blebbistatin.\textsuperscript{37,38} Finally,
FN-matrix assembly was also prevented by the presence of RGDS demonstrating that integrin
activation plays a fundamental role in FN-matrix assembly by human Mks.\textsuperscript{39} Taken together these
results indicate that Mks can control FN assembly with integrins and Mk cytoskeleton playing a
central role in this regulatory mechanism.\textsuperscript{40} Further, specificity of the matrix substrate is
fundamental for this process as Mks can support FN assembly only upon adhesion on collagen type
I and not on FBG.

Interestingly, FN is a major component of bone marrow ECM and may modulate homing of
hemopoietic progenitor cells\textsuperscript{41} as well as organization and composition of bone marrow ECMs and
cell-matrix adhesion sites.\textsuperscript{40} The present work shows a new role for Mks as promoter of matrix
deposition and remodeling within bone marrow environment with particular emphases to type I
collagen rich osteoblastic niche.

Transglutaminases (TGs) are a widely distributed group of enzymes that catalyse the post-
translational modification of proteins by the formation of isopeptide bonds. TGs have been shown
to bind and cross-link a number of extracellular proteins, in particular fibronectin, for which it has a
high binding affinity.\textsuperscript{42} The physiological implications related to matrix protein crosslinking
indicate that its function is not only to stabilize these proteins, i.e. increasing their proteolytic,
chemical and mechanical resistance, but also to facilitate cell adhesion and cell motility.\textsuperscript{25}
Interestingly, a recent research by Jayo et al. reported that FXIII-A represents the only detectable source of TG activity by protein analysis in platelets. Here we demonstrate that, among all the TGs, FXIII-A is the most expressed by human Mks at both mRNA and protein levels. Moreover FXIII-A was constitutively active in Mks, as cross-linking activity was detected by biotinylated cadaverine incorporation in different intracellular substrates. Additionally, TGase activity was shown to be associated to Mk plasma membrane as revealed by analysis of cross-linked biotinylated cadaverine to adsorbed FN matrix in ELISA assay. All together these results underline that active FXIII-A may represent an important biochemical modulator of different Mk functions related to its enzymatic activity at both intracellular and membrane sites. In particular, here we analyzed how FXIII-A activity impact Mk spreading and FN matrix assembly on type I collagen. FXIII-A was shown to regulate Mk spreading and stress fibers formation on type I collagen as a significant decrease of these processes was observed upon incubation with TG inhibitors. Consistently, FXIII-A co-localized with FN on the plasma membrane of spread Mks and FN matrix assembly by Mks on type I collagen was inhibited upon incubation with TG inhibitor iodoacetamide. These results confirmed an important role of FXIII-A in stabilizing Mk-type I collagen interaction through FN fibrillogenesis.

The BM microenvironment has been shown to undergo continual remodeling with a balance between ECM deposition and destruction. Interestingly, our data represent a sort of mirror image of previous works that demonstrated that local secretion of proteases induce HSCs mobilization from the BM niche by altering HSC-stromal cell interaction and that production of MMP-9 by Mks is one of the mechanisms by which mature MKs free themselves from the BM matrix and travel to the vascular niche. This study provides important new elements in the understanding of the regulatory pathways for Mk-matrix interactions within bone marrow environment. In particular, our results demonstrate that fibronectins and FXIII-A modulate Mk spreading on type I collagen by promoting matrix assembly.
In conclusion, this work opens new prospective in the study of diseases, such as primary myelofibrosis or MYH9-related thrombocytopenia, characterized by a defect of Mk-matrix interactions within the bone marrow environment, whose origin is still matter of debate.

Acknowledgements

We thank P. Vaghi (“Centro Grandi Strumenti”, University of Pavia) and D. Picenoni (Politecnico of Milan) for their technical assistance in confocal and scanning electron microscopy studies. We thank G. Viarengo for technical assistance in flow cytometry studies. We thank C. Di Buduo, V. Abbonante and S. Badalucco (Department of Biochemistry, University of Pavia) for assistance in performing experiments. We also thank V. Rosti for critical review of the paper. This paper was supported by Cariplo Foundation (2006.0596/10.8485) to AB, FAR 2008 to LV, Banca del Monte di Lombardia Foundation to LV and AB, PRIN (2007TWL3MX_003) to MET. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contribution and Conflict of interest

AM, CG, PR and LV performed the research; AM, CB, MET and AB designed research, interpreted and analyzed the data; CP provided essential reagents; RM analyzed data; AM and AB wrote the paper. No author of this paper has a conflict of interest.

References


Figure Legends

Figure 1: Human Mks express cFN isoforms.

CD61+ megakaryocyte at 13 days of culture were separated by immunomagnetic beads technique and total cellular RNA was extracted. (A) RT-PCR demonstrated expression of cFN (FN1 gene), EDA (+) fibronectin and EDA (-) spliced fibronectin mRNA sequence, EDB (+) fibronectin and spliced EDB (-) isoform. (B) Mks were permeabilized and stained in IF with a polyclonal antibody against cFN (red) and (C) a monoclonal antibody against EDA isoform (green). Scale bars are=20μm and 10μm respectively. (D) cFN relocated to Mk plasma membrane upon activation with thrombin (1U/ml) (upper panels) or omitting thrombin (lower panels) as revealed by IF with polyclonal antibody against cFN (red). Nuclei were stained with Hoechst 33288 (blue). Scale bar=10μm.

Figure 2: cFN modulates Mk adhesion on collagen type I, but not on fibrinogen.

Mature Mks were differentiated from human cord blood CD34+ cells as described in methods. Mature Mks were then incubated on type I collagen or FBG coated coverslips for 16 hours at 37°C in an atmosphere of 5% CO2 in the presence of TPO. (A) After 3 and 16 hours of incubation coverslips were fixed and Mk spreading and PPF were evaluated as described in methods section. (B) Representative image of Mk forming proplatelet upon FBG adhesion. Cells were stained with an anti α-tubulin antibody (green) and Hoechst 33288 for nuclear staining (blue). Scale bar=20μm. (C) After 3 hours incubation coverslips were fixed, permeabilized and stained with a polyclonal antibody against cellular fibronectin. Subcellular localization of cFN (red) was analyzed by confocal microscopy. Scale bars=10μm. (D) Immunoprecipitation of released fibronectin in supernatants harvested after 3 hours of Mk adhesion to type I collagen (lane 2) or FBG (lane 3). FN from human plasma was used as positive control (lane 1), while unconditioned Stem Span medium was used as negative control (lane 4). Actin was revealed by western blotting in adherent cells on...
both matrices to ensure the same number of cells. (E) Scanning electron microscopy of Mk spread on type I collagen after 3 hour incubation, in the right panel immunogold staining with polyclonal fibronectin antibody is shown. Arrows indicate fibronectin exposure on Mk membrane. Scale bar=1μm. * p<0.05

**Figure 3: Integrin engagement in Mk adhesion to type I collagen.**

(A) Subcellular localization of cFN (red), CD49e (green, upper panel), CD49b (green, lower panel) in Mk spread on type I collagen. Co-localization between cFN and receptor components was analyzed by merging images obtained in green and red channels in each confocal optical section (composite). In the right panels image magnification showing proteins co-localization on Mk plasma membrane (yellow staining, arrows). All slides were also incubated with Hoechst 33288 for nuclear staining (blue). Scale bar=10μm. (B) Effect of RGDS, CD49e, CD49d antibodies and their combination on Mk spreading upon incubation for sixteen hours. Results are reported as percentage of inhibitor-treated Mks adherent to collagen type I compared to Mks treated with PBS alone, and are the mean ± S.D. of three different experiments. * p<0.05

**Figure 4: FN assembly by Mks adherent to type I collagen.**

The ability of Mk to assembly pFN was evaluated on type I collagen and FBG as described in methods section. (A) Assembly of FITC labelled FN (green) by CD61+ Mks (red) adherent on type I collagen and FBG (Scale bar=10μm). In parallel experiments staining of fibrillar pFN with a polyclonal antibody in Mks plated on type I collagen and FBG (Scale bar=50μm). Mks adherent on type I collagen and FBG were removed after deoxycholic acid treatment (DOC) and DOC-insoluble pFN fibrils were stained with a polyclonal antibody anti-FN (red). Nuclei were always counterstained with Hoechst 33288 (blue). Scale bar=20μm. (B) The presence of DOC-insoluble assembly of pFN was also revealed by immunoblot analysis using a monoclonal anti-FN, clone
IST-4. In a parallel sample, cells were scraped and proteins separated by SDS-PAGE and then stained with anti-beta actin to ensure the same number of cells in the experiment. (C) Evaluation of cytoskeleton and integrin roles in FN matrix assembly by Mks in samples treated with RGDS or blebbistatin with respect to control. FN matrix after DOC-treatment was stained with a polyclonal antibody anti-FN and visualized in IF. Scale bar=20μm. (D) The presence of endogenous EDA+ FN during pFN deposition and assembly was evaluated as above described in IF, staining were performed using both the polyclonal antibody against FN and monoclonal anti-EDA fibronectin (Scale bar=20μm).

**Figure 5: Mks express and synthesize FXIII-A.**

(A) RT-PCR analysis of different TGs by human Mks. The amplification products were resolved on agarose gel and visualized by ethidium bromide staining. Actin amplification was used as control. (B) Western blot analysis of FXIII-A in human Mks. FXIII from human plasma was used as positive control. (C) Analysis of intracellular TG activity of FXIII in human Mks. Mk lysates were incubated with biotinylated cadaverine in the absence or presence of EDTA or Ca²⁺, and resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis and blotted with peroxidase-conjugated avidin. TG activity was completely reverted upon calcium sequestering with EDTA. (D) Extracellular TG activity was demonstrated by ELISA assay of cadaverine incorporation into pFN upon adhesion of Mks for sixteen hours and DOC-treatment. TG activity was significantly reduced by incubation with TGs inhibitors such as iodoacetamide or cystamine (10μM each). *p<0.05

**Figure 6: FXIIIa modulates Mk spreading and FN assembly on type I collagen.**

(A) Co-localization, during FN fibrillogenesis, of FXIII-A (green) and pFN (red) at membrane level of Mks adherent on type I collagen. Scale bar=10μm. (B) Mk spreading on type I
collagen was modulated by TGs activity: analysis of Mk CD61+ exhibiting stress fibers in the presence of TGs inhibitors such as iodoacetamide (IAA), monodansylcadaverine (MDC) and cystamine (CYS). Results are reported as percentage of inhibitor-treated Mks adherent to collagen type I compared to Mks treated with PBS alone, and are the mean ± S.D. of three different experiments. (C) Effects of IAA treatment on Mk spreading and FITC-labelled FN assembly on type I collagen. Cells were stained with TRITC-phalloidin and incubated with 25 μg/ml of FITC-FN and IF performed as described in methods section. Nuclei were counterstained with Hoechst 33288 (blue). Scale bar=50μm. *p<0.05
FIGURE 3

A

B

% of CD61+ cells exhibiting stress fibers relative to control

CTRL | RGDS | ANTI-CD49e | ANTI-CD49d | ANTI-CD49e + ANTI-CD49d

0  | 40  | 60  | 80  | 120
FIGURE 6

A. FN + FXIII-A + Hoechst

+ 25 μg/ml pFN

B. % of CD61+ cells exhibiting stress fibers relative to control

CTRL  IAA  MDC  CYS

C. Type I collagen + Iodoacetamide 10 μM

TRITC-Phalloidin + Hoechst

Anti FN + Hoechst

+ 25 μg/ml pFN
Megakaryocyte-matrix interaction within bone marrow: new roles for fibronectin and factor XIII-A

Alessandro Malara, Cristian Gruppi, Paola Rebuzzini, Livia Visai, Cesare Perotti, Remigio Moratti, Cesare Balduini, Maria Enrica Tira and Alessandra Balduini